


MiR-375-mediated suppression of engineered coxsackievirus B3 in pancreatic cells

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Coxsackievirus B3 (CVB3) has potential as a new oncolytic agent for the treatment of cancer but can induce severe pancreatitis. Here, we inserted target sequences of the microRNA miR-375 (miR-375TS) into the 5' terminus of the polyprotein encoding sequence or into the 3'UTR of the CVB3 strain rCVB3.1 to prevent viral replication in the pancreas. In pancreatic EndoC-βH1 cells expressing miR-375 endogenously, replication of the 5'-miR-375TS virus and that of the 3'-miR-375TS virus was reduced by 4×10^3 -fold and 3.9×10^4 -fold, respectively, compared to the parental rCVB3.1. In colorectal carcinoma cells, replication and cytotoxicity of both viruses were slightly reduced compared to rCVB3.1, but less pronounced for the 3'-miR-375TS virus. Thus, CVB3 with miR-375TS in the 3'UTR of the viral genome may be suitable to avoid pancreatic toxicity.

Keywords: cancer therapy; colorectal cancer; coxsackievirus B3; microRNA; oncolytic virus

Coxsackievirus B3 (CVB3) is a nonenveloped virus of the *picornaviridae* family, with an icosahedral capsid and a positive-sense, single-stranded RNA genome with a length of about 7.4 kb [1]. It has a short replication cycle of only 6–8 h and produces huge amounts of progeny virus [2]. In humans, the virus infects the gastrointestinal tract and typically induces local intestinal disease with mild flu-like symptoms [3]. Under certain circumstances, CVB3 can pass the local barrier of the intestine and spread *via* the bloodstream to other organs where it replicates to high titers and induces inflammatory disease, most commonly myocarditis [4,5], pancreatitis [6], and aseptic meningoencephalitis [7,8]. CVB3 infection usually resolves by itself, but occasionally it progresses to severe disease which can have a fatal outcome. There are many different strains

of CVB3 with different phenotypic characteristics, leading to different courses of infection [8–12].

In 2012, the oncolytic activity of CVB3 was first described by Miyamoto *et al.* [13]. Intratumoral injection of the CVB3 Nancy strain suppressed growth of subcutaneous human non-small-cell lung carcinomas in mice. However, the virus also induced mild hepatitis, myocarditis [13], and later pancreatitis was also observed [14]. More recently, we reported growth inhibition of subcutaneous human colorectal carcinomas in mice following intratumoral injection of three different CVB3 strains [15]. Two of the three strains (31-1-93 and Nancy) also induced severe pancreatitis and myocarditis [15]. A third study reported strong oncolytic activity in endometrial cancer *in vivo* using the CVB3 strain CV-B3/2035A which was

Abbreviations

CVB3, coxsackievirus B3; miR-TS, microRNA target sites.

isolated from a throat swab of a patient with head, foot, and mouth disease. The virus did not induce significant treatment-related toxicity and mortality in nude mice, despite the fact that the virus was detected at moderate levels in the heart, lung, and kidney [12].

MicroRNAs (miRs) are small noncoding RNAs which are endogenously expressed in eukaryotic cells and processed to an ~ 22 bp long RNA duplex from a premature precursor with hairpin structure containing an imperfectly base-paired stem [16,17]. One strand of these mature miR bind to cognate miR target sequences (miR-TS) in cellular mRNAs, inducing post-transcriptional repression of protein synthesis [18]. Many miRs are cell-, tissue-, and organ-specifically expressed, and the cellular abundance of the miR varies greatly [19,20]. It has been shown that by insertion of corresponding miR-TS into virus genomes, replication of the virus can be suppressed in a tissue-specific manner [21–24]. To optimize the suppression, two to four copies of a miR-TS or a combination of different miR-TS are commonly inserted into the viral genome [23,24]. Moreover, unlike cellular genes, miR-TS in oncolytic viruses are completely complementary to the cognate miR which enables endonucleolytic cleavage of the miR-TS by argonaut 2 [25] and correspondingly increases the efficiency of target suppression [23,26]. In picornaviruses, the site of miR-TS insertion within the viral genome seems to be a further aspect critically influencing virus suppression. Previous published studies successfully inserted miR-TS within the 5' and 3'UTRs of the viral genome [14,23,27]. However, it has also been shown that certain sections within the 5'UTR and the 3'UTR do not tolerate miR-TS insertion [28].

Here, we report that insertion of miR-TS of the pancreas-specifically expressed miR-375 into the 5' terminus of the CVB3 polyprotein encoding sequence or into the 3'UTR immediately downstream of the stop codon of the polyprotein is well tolerated by the virus. Both viruses replicate poorly and lose their cytotoxicity in pancreatic cells expressing miR-375, while retaining their replication competence in the targeted colorectal cancer cells. However, when comparing the two viruses, CVB3 containing the miR-TS in the 3'UTR showed improved performance.

Materials and methods

Cell lines

HEK293T (human embryonic kidney) cells were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM;

PAA Laboratories GmbH, Cölbe, Germany) supplemented with 1% sodium pyruvate, 1% L-glutamine, 10% FCS (c.c. pro GmbH, Oberdorla, Germany), and 1% each of penicillin and streptomycin (P/S; Sigma, Munich, Germany). HeLa cells (human cervical carcinoma) were cultured in modified Eagle's medium (MEM) complete medium, which consists of MEM supplemented with 2 mM L-glutamine, 5% FCS, 1% NEAA, 0.02 M Hepes buffer, and 1%P/S. EndoC-βH1 cells [29] were cultured in DMEM, low glucose (1 g·L⁻¹), containing 2% BSA (fraction V), 10 mM nicotinamide, 5.5 μg·mL⁻¹ transferrin, 6.7 ng·mL⁻¹ sodium selenite, 50 μM β-mercaptoethanol, 100 U·mL⁻¹ penicillin (P), and 100 μg·mL⁻¹ streptomycin. Colorectal carcinoma cell lines DLD1 and Colo320 were grown in RPMI 1640 supplemented with 10% FCS, 1% penicillin-streptomycin, 1% L-glutamine, and 1 mM Na-pyruvate (Invitrogen, Karlsruhe, Germany). Caco-2 cells were cultured in DMEM supplemented with 10% FCS, 1% P/S, 1% 2 mM L-glutamine, and 2% sodium pyruvate.

Plasmids

For the construction of CVB3 cDNA plasmids bearing miR-TS, the plasmid pMKS1 [2], kindly provided by Lindsay Whitton (Scripps Research Institute, La Jolla, CA, USA) was used. It contains the sequence of the CVB3 clone H3 [11], which was modified by insertion of a *Sfi*I site and an artificial protease cleavage site immediately downstream of the CVB3 polyprotein initiation codon. To insert miR-375TS into the 3'UTR of CVB3, three tandem copies of the miR-375TS and flanking sequences were generated by gene synthesis (Thermo Fisher Scientific, GENEART GmbH, Regensburg, Germany) subcloned and inserted into pMKS1 via *Cl*aI/*St*uI restriction sites. The plasmid was named pMKS1-3'-375TS (+). To insert miR-375TS into the 5' terminus of the CVB3 polyprotein coding region, the synthesized miR-375TS and flanking sequences were subcloned and inserted in frame via *Asi*SI and *Pme*I, which were inserted into the *Sfi*I site of pMKS1. The resulting plasmid was termed pMKS1-5'-375TS(+). The same procedure was used to insert three copies of miR-375TS in the inverse orientation into the 3'UTR and at 5' terminus of the CVB3 polyprotein coding region. The plasmids were named pMKS1-3'-375TS(-) and pMKS1-5'-375TS(-), respectively. Two control plasmids, pMKS1-3'-39TS(+) and pMKS1-5'-39TS(+), were also generated as described above. They each contain three copies of miR-TS of cel-miRNA-39 from *Caenorhabditis elegans*, which has no homologous miR in mammals [30], in the sense orientation in the 3'UTR and at the 5' terminus of the CVB3 polyprotein coding region, respectively. The plasmids pCMV-MIR-375 expressing miR-375 and pCMV-MIR-216a expressing miR-216a were purchased from OriGene (OriGene Technologies Inc., Rockville, MD, USA).

Viruses

rCVB3.1, CVB3-375TS(3+), CVB3-375TS(3−), CVB3-375TS(5+), CVB3-375TS(5−), CVB3-39TS(5+), and CVB3-39TS(3+) were generated by transfection of plasmids pMKS-1, pMKS1-3′-375TS(+), pMKS1-3′-375TS(−), pMKS1-5′-375TS(+), pMKS1-5′-375TS(−), pMKS1-3′-39TS(+), and pMKS1-5′-39TS(+) into 80% confluent HEK293T cells using Polyethylenimine Max (Polysciences, Inc., Warrington, PA, USA) and amplified as described [15], and then stored at −80 °C until use.

Growth curves

HeLa cells were seeded in 24-well plates and infected the next day at an MOI of 0.01 with the CVB3 variants at a confluence of 90% in 500 µL of MEM. Cells were incubated for 2 h at 37 °C and afterward 500 µL of MEM complete medium was added. After 2, 4, 8, 24, and 48 h the samples were frozen and thawed twice, centrifuged, and the supernatant was analyzed by plaque assay on HeLa cell monolayers as described below. Plaques sizes were measured with a caliper.

Viral plaque assay

HeLa cells were cultured in 24-well cell culture plates as confluent monolayers. After 24 h, medium was removed, and cells were incubated for 30 min with serial ten-fold dilutions of supernatants, harvested from virus-infected cell lines after three freeze/thaw cycles and overlaid with agar containing MEM. Three days later, the cells were stained with 0.5% MTT/PBS (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma-Aldrich, Steinheim, Germany).

Transfection of MiR expression plasmids

HEK293T cells were seeded in 6-well plates and 24 h later, at a confluence of 70–90%, they were transfected with miR expression plasmids using Polyethylenimine MAX 40K.

XTT cell viability assay

XTT assay was carried out as suggested by the supplier (Roche Diagnostics, Mannheim, Germany). Briefly, EndoβC-H1, DLD-1 cells and Caco-2 cells were seeded in 96-well plates and infected at an MOI of 100 (DLD1 cells), 1 (EndoβC-H1), or 0.01 (Caco-2 cells) with recombinant CVB3 when they reached a confluence of 90%. Cell viability was measured at 144 h (DLD1 cells), 72 h (Caco-2 cells), and 48 h (EndoβC-H1) after infection with recombinant CVB3 with the TriStar² LB 942 Modular Multimode Microplate Reader (Berthold Technologies, Bad Wildbad,

Germany) or Infinite M200 Pro (Tecan, Männedorf, Switzerland).

Quantification of miR-375

Total RNA from cells was isolated with TRIZOL (Life Technologies GmbH, Darmstadt, Germany) according to the manufacturer's instructions. Expression levels of miR-375 were determined by utilizing the TaqMan gene expression master mix and specific FAM-tagged TaqMan gene expression assays for hsa-miR-375 (assay ID: 000564), both from Life Technologies. Equal loading of RNA was determined by the measurement of snU6RNA expression as described [31]. Real-time PCR was performed in a FX96 Real-Time System combined with a C1000 Thermal Cycler (Bio-Rad, Hercules, CA, USA). The PCR reactions were carried out in triplicates, and expression values of miR-375 were determined by the $\Delta\Delta C_t$ calculation method.

Western blot analysis

Cells were treated with lysis buffer (20 mM TRIS/HCl, pH 8.0, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% protease inhibitor cocktail) (Sigma-Aldrich, Taufkirchen, Germany) and 1% phosphatase inhibitor cocktail (Calbiochem, San Diego, CA, USA). Protein concentration was measured by a BCA assay (Thermo Fisher Scientific, Waltham, MA, USA). Cell extracts were separated by SDS/PAGE and immunoblotted as described [32]. Primary antibody anti-γ-tubulin was from Sigma-Aldrich and anti-eIF4G, cleaved caspase 3, and anti-PARP from Cell Signaling Technology (Danvers, MA, USA). The monoclonal anti-VP1 was generated against VP1 from CVB5 strain Faulkner. For detection of CVB3 VP1, γ-tubulin, PARP, cleaved caspase 3, and eIF4G, the membrane was blocked with 5% dry milk/PBS-T and subsequently incubated at 4 °C overnight with the respective antibodies. After washing three times with PBS-T, the membrane was incubated with goat anti-mouse and anti-rabbit IgGs conjugated to horseradish peroxidase (Bio-Rad) in 5% dry milk/PBS-T for 1 h. Magic MarkXP (Thermo Fisher Scientific) was used as a molecular weight marker to determine size of detected proteins after western blotting. Chemiluminescence was performed using the Supersignal West Pico Substrate (Thermo Fisher Scientific) and detected with Imager 600 from GE Healthcare (Chalfont St Giles, UK).

Statistics

Statistical analysis was performed with GRAPHPAD PRISM 5.03 (GraphPad Software, Inc., La Jolla, CA, US). Results are expressed as means ± SEM. Statistical significance was determined by an unpaired Student's *t*-test. All differences were considered statistically significant at a *P* of < 0.05.

Results

Expression of miR-375 in pancreatic and colorectal cell lines

The effectiveness of miR-mediated suppression of oncolytic viruses strongly depends on the level of miR expression. In fact, the miR must be highly expressed in tissues where the virus is to be suppressed, while its expression must be low or absent in the targeted cancer cells. The miR-375 is the most abundantly expressed miR in the pancreas and is absent or only expressed at very low levels in other tissues [19,33], making it a promising candidate for development of miR-targeted pancreas-attenuated oncolytic CVB3. Using RT-PCR analysis, we confirmed high expression of miR-375 in the murine pancreas and found similar miR-375 expression levels in the pancreatic cell line EndoC- β H1. In contrast, much lower miR-375 expression was detected in the colorectal carcinoma cell lines DLD1 (220-fold), Caco-2 (1200-fold), and Colo320 (1 000 000-fold), as well as the miR-375 expression levels in the CVB3 producer cell lines HeLa and 293T were about 10^4 - and 10^5 -fold lower than in EndoC- β H1 cells and the murine pancreas (Fig. 1A). Thus, we chose miR-375 for further investigations.

Construction of miR-375TS containing CVB3

To investigate whether replication of miR-375-targeted CVB3 can be inhibited by miR-375, we inserted three copies of a target site with complete complementary to the miR-375 (miR-375TS) into the cDNA of the CVB3 variant rCVB3.1 [2]. The miR-375TSs were inserted into the virus genome, either immediately downstream of the start codon at the 5' terminus of the CVB3 polyprotein coding region or immediately downstream of the stop codon in the 3'UTR, in both cases embedded in stuffer sequences and in both the forward (+) and inverted orientation (–) (Fig. 1B). Both orientations were created to elucidate whether miR-375-induced inhibition differs when the plus- or the minus-strand replication intermediate of CVB3 is targeted by miR-375. As controls, two recombinant CVB3s were produced containing three copies of miR-39TS of miR cel-miR-39 from *C. elegans*, which has no homologue in mammals, in the above-mentioned sites of the viral genome, in each case in the plus strand of the CVB3 genome (Fig. 1B).

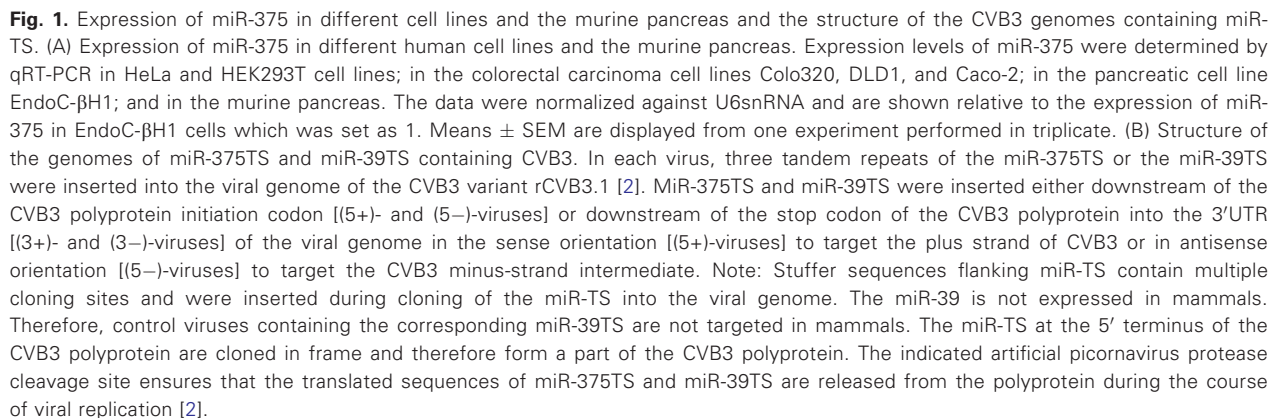
After production of viruses in HEK293T and HeLa cells, the size of virus plaques was investigated. For CVB3 with miR-TS in the 3'UTR, we observed that plaque size of CVB3-375TS(3–) was similar to that of

the parental rCVB3.1, whereas the plaques were slightly smaller in CVB3-375TS(3+) and in the control virus CVB3-39TS(3+). Differences in plaque sizes were also observed for CVB3 containing the miR-TS in the 5' terminus of the viral polyprotein coding region, whereas CVB3-375TS(5+) and the control virus CVB3-39TS(5+) showed similar plaque size as rCVB3.1, and CVB3-375TS(5–) showed distinctly smaller plaques (Fig. 2A). To further compare growth kinetics of miR-TS viruses, we next generated growth curves in highly susceptible HeLa cells for each virus over a 48-h investigational period. The 3'UTR CVB3-375TS(3–) and the control virus CVB3-39TS(3+) showed slightly delayed, and CVB3-375TS(3+) moderately delayed, growth within the first 24 h after infection compared to rCVB3.1. However, after 48 h the titers of the three miR-375TS viruses were similar to those of rCVB3.1, indicating the decrease in growth rate was limited to the first 24 h. Similar was seen for viruses with miR-TS at the 5' terminus of the CVB3 polyprotein coding region. All miR-TS viruses of this group showed moderately reduced growth within the first 24 h after infection. However, while 48 h after infection the differences in virus titers between CVB3-375TS(5+), the control virus CVB3-miR-39TS(3+), and the parental rCVB3.1 disappeared, the titers of CVB3-375TS(5–) remained about one \log^{10} below the titers of rCVB3.1 (Fig. 2B).

Taken together, the data indicate that insertion of miR-TS into the 3'UTR or into the 5' terminus of the polyprotein coding region can negatively affect the growth of CVB3. In particular, the CVB3 variant containing miR-375TS in the viral minus strand at the 5' terminus of the CVB3 polyprotein coding region showed greatly impaired growth.

CVB3-375TS(3+) is inhibited more strongly than CVB3-375TS(5+) by transiently transfected miR-375 in HEK293T cells

To elucidate whether the CVB3-375TS can be inhibited by miR-375 and whether there are differences between the constructs, next we transfected HEK293T cells with miR-375 and infected the cells 24 h later at an MOI of 0.1 with miR-375TS containing CVB3 or with the miR-39TS control viruses. As detected by real-time RT-PCR, miR-375 was highly expressed at the time point when the cells were infected with CVB3 (Fig. 3A). Plaque assays carried out 24 h after CVB3 infection showed there was no inhibition of CVB3-375TS(5–) and CVB3-375TS(3–), indicating that the negative-strand replication intermediate of virus could not be targeted by miR-375. In contrast, both



Having demonstrated inhibition of CVB3-375TS(3+) and CVB3-375TS(5+) in cells expressing exogenously

introduced miR-375, we next analyzed these viruses for replication in pancreatic EndoC- β H1 cells, which express miR-375 endogenously. EndoC- β H1 cells were infected at an MOI of 1 and analyzed 24 h later. As shown in Fig. 4A, the titers of CVB3-375TS(3+) and CVB3-375TS(5+) were 7700-fold and 407-fold lower compared to the miR-39TS control viruses and even 39 000-fold and 4000-fold lower than the parental rCVB3.1, respectively. These data were confirmed by the measurement of CVB3 VP1 expression. As shown

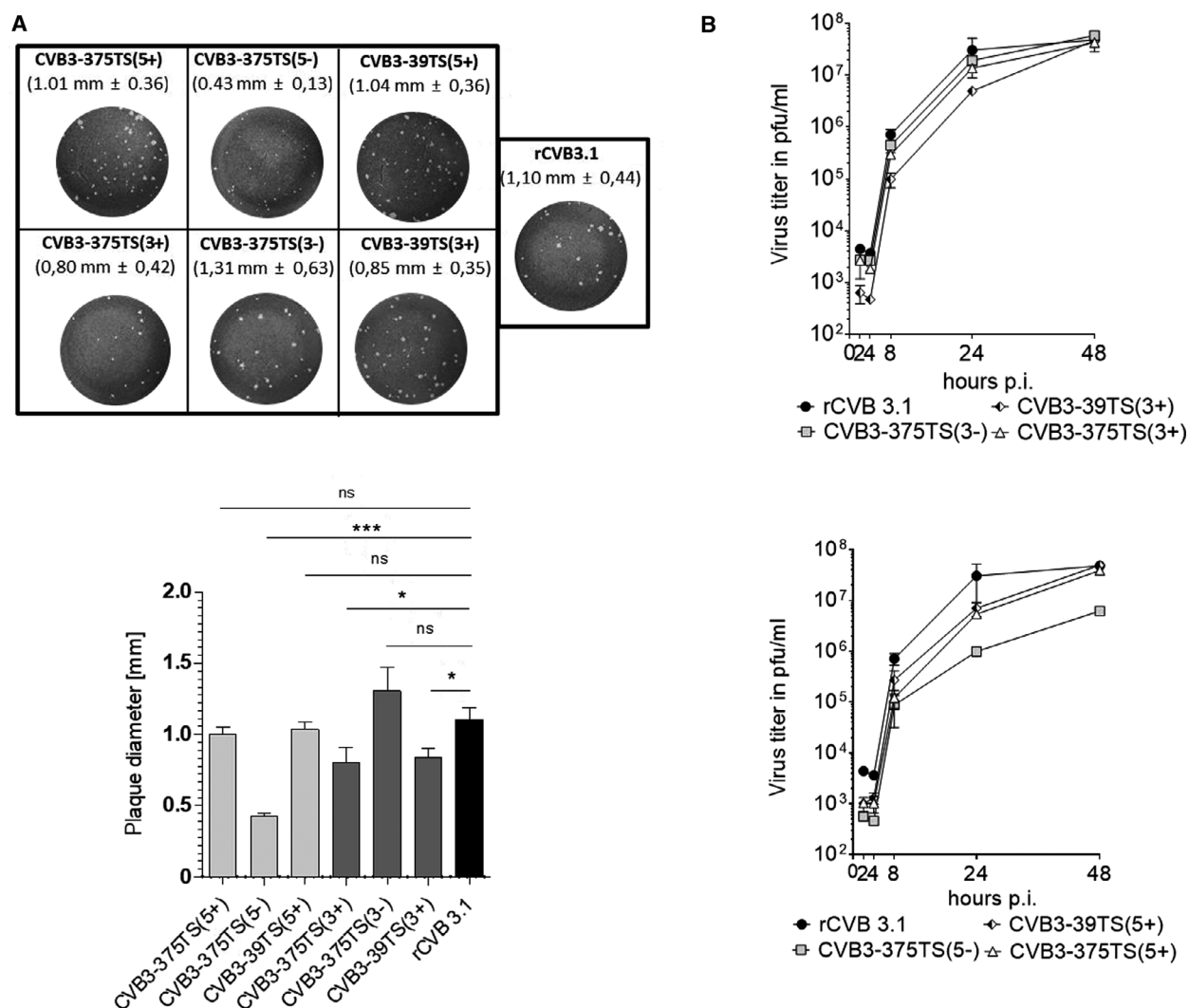


Fig. 2. Plaque size and growth kinetics of miR-TS-bearing CVB3 and parental CVB3 variant rCVB3.1. (A) Plaque sizes. Plaque sizes of indicated miR-TS viruses were determined by plaque assays on HeLa cell monolayers. Upper panel shows representative images of viral plaques (white dots) with an average plaque diameter of 20–45 counted plaques ± SEM determined in two experiments. Lower panel presents the graphical representation of plaque diameter. The mean values ± SEM are displayed from two experiments and 20–45 counted plaques. Significance: * $P < 0.05$, *** $P < 0.001$; n.s. not significant. (B) Growth curves. HeLa cells were infected at an MOI of 0.01 with indicated miR-TS CVB3 or rCVB3.1, and the generation of virus progeny was measured by plaque assay 2, 4, 8, 24, and 48 h later. Upper graph shows recombinant CVB3 with miR-TS in the 3'UTR, and lower graph shows recombinant CVB3 with miR-TS at the 5' terminus of the CVB3 polyprotein encoding sequence. Results are shown as mean values ± SEM and reflect two experiments, one with two and one with four replicates.

by western blotting, VP1 expression was not detected in cells infected with miR-375TS-containing viruses, whereas miR-39TS control viruses showed abundant VP1 expression (Fig. 4B). In accordance with the lack of VP1 expression and strongly reduced virus replication, EndoC-βH1 cells infected with CVB3-375TS(5+) and CVB3-375TS(3+) showed lower expression levels of the CVB3 2A protease target protein eIF4G and apoptosis markers PARP and caspase 3 compared to

those in the uninfected control cells (Fig. 4B). Moreover, both CVB3-375TS(3+) and CVB3-375TS(5+) did not induce cytotoxicity in EndoCβ-H1 cells, whereas the control viruses induced pronounced lytic infection (Fig. 4C). The data indicate that both miR-375TS viruses were efficiently suppressed by endogenously expressed miR-375. However, determination of virus titers reveals a distinctly stronger suppression of CVB3-375TS(3+).

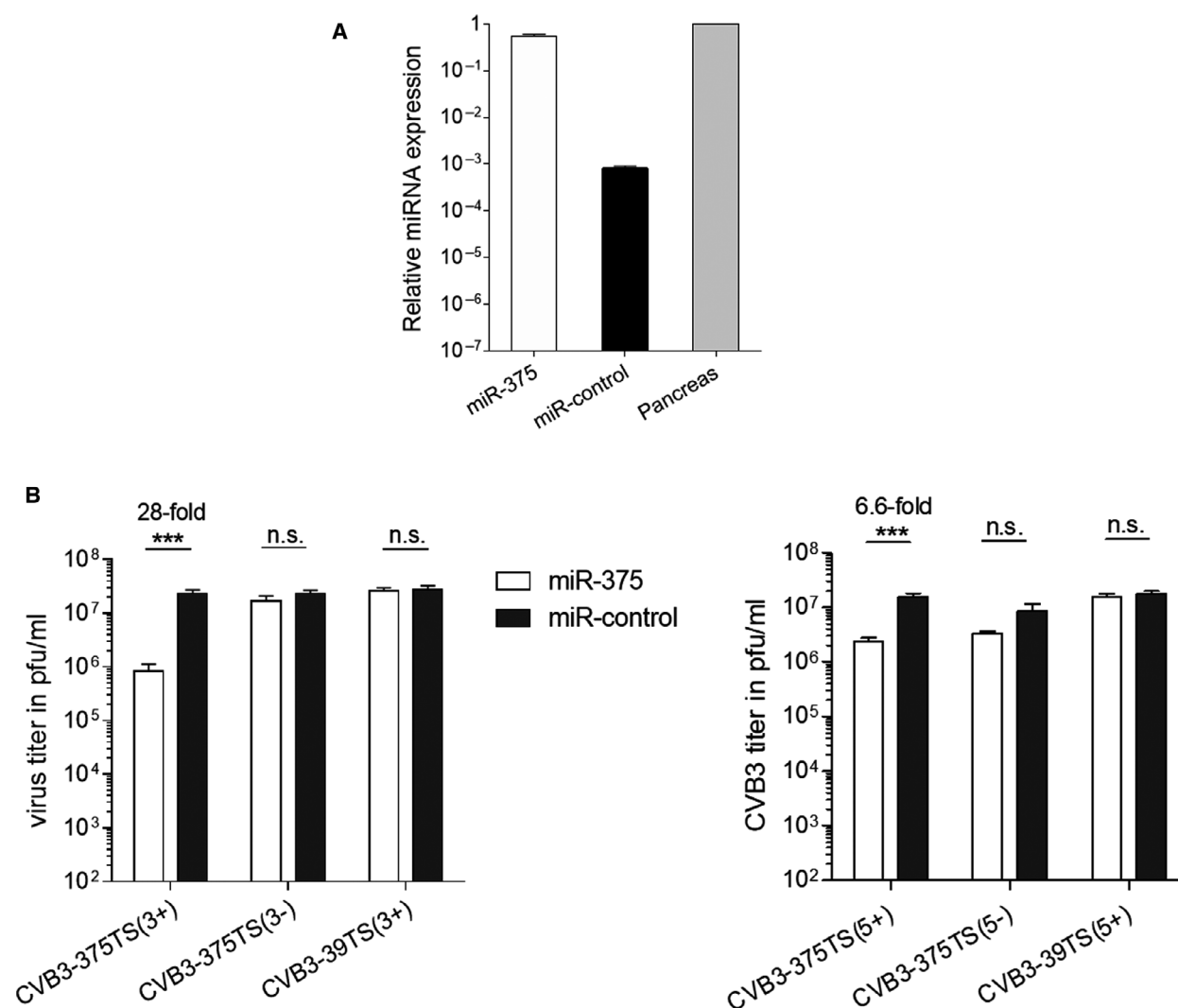


Fig. 3. Silencing of recombinant miR-375TS containing CVB3 by miR-375. (A) Expression of miR-375 after transfection of HEK293T cells. HEK293T cells were transfected with a plasmid expressing miR-375 or control plasmid (miR-control) expressing the miR-216a. Twenty-four hours later, expression of miR-375 was determined by real-time RT-PCR. The data were normalized against U6snRNA and are shown relative to the expression of miR-375 in murine pancreas, which was arbitrarily set to 1. Mean values \pm SEM are displayed from one experiment performed in triplicate. (B) Silencing miR-375TS containing CVB3 by miR-375. HEK293T cells were transfected as in 3a and infected 24 h later with the indicated recombinant CVB3 at an MOI of 0.1. Virus replication was analyzed 24 h later by plaque assay. Left diagram—recombinant CVB3 with miR-TS in the 3'UTR. Right diagram—recombinant CVB3 with miR-TS at the 5'-terminus of the CVB3 polyprotein encoding sequence. Mean values \pm SEM are displayed from two experiments performed, each in triplicate. Significance: *** $P < 0.001$; n.s. not significant.

CVB3-375TS(3+) replicates better and induces more cytotoxicity in colorectal carcinoma cell lines than CVB3-375(5+)

Finally, we investigated whether insertion of miR-375TS into the viral genome affects replication and cytotoxicity of respective viruses in colorectal carcinoma cell lines. Therefore, the susceptible colorectal carcinoma cell line Caco-2 was infected with an MOI of 0.01 and the more resistant cell line DLD1 with an

MOI of 100 of CVB3-375TS(3+) and CVB3-375TS(5+) and the control viruses CVB3-39TS(3+), CVB3-39TS(5+), and rCVB3.1. Determination of virus titers by plaque assay 48 h after infection showed that in both cell lines, the replication of CVB3-375TS(3+) was slightly lower than the replication of parental rCVB3.1 (1.8-fold in Caco-2 and 4.1-fold in DLD-1). A distinctly stronger reduction of virus replication was observed for CVB3-375TS(5+) when compared to

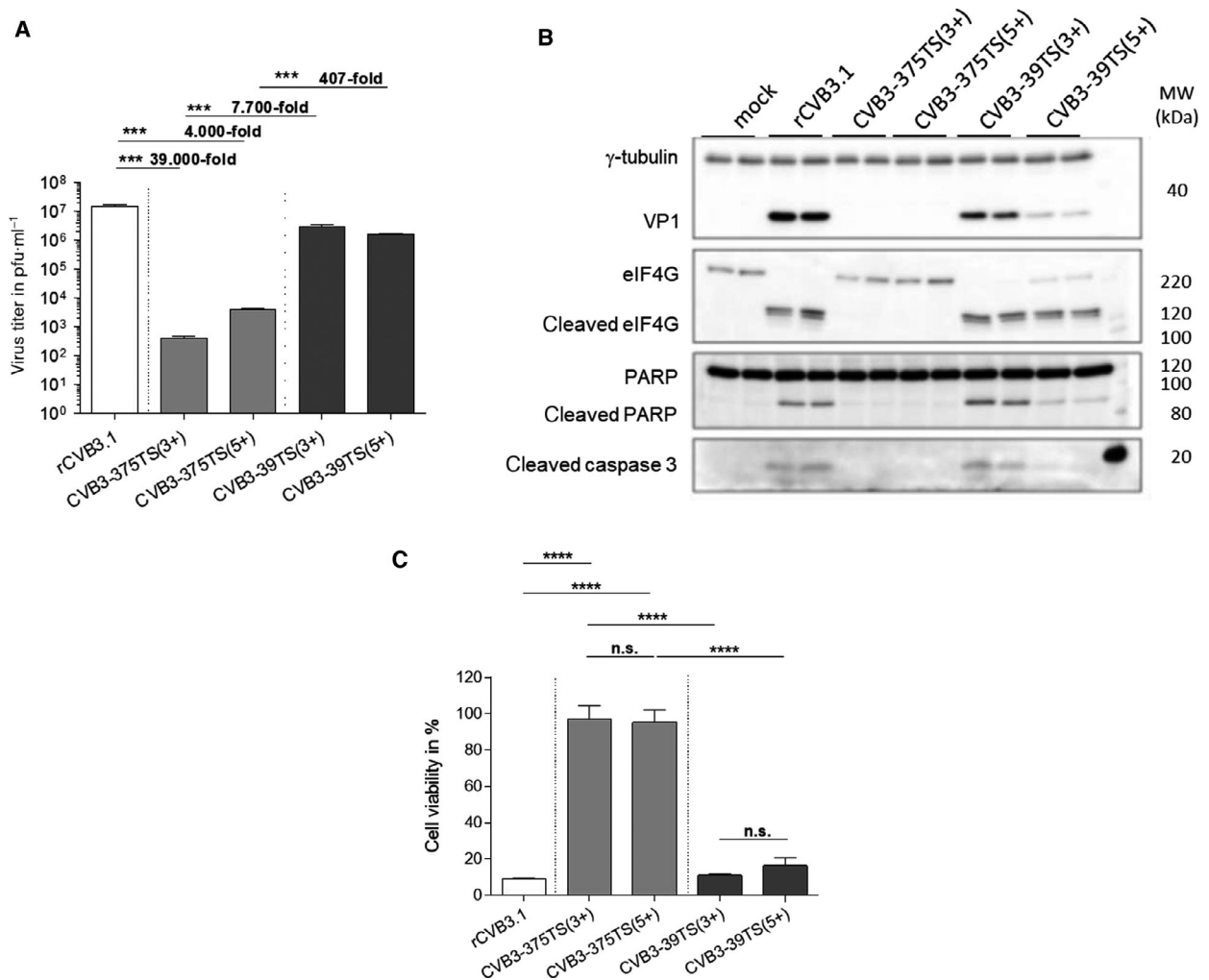


Fig. 4. Suppression of miR-375TS-containing CVB3 in pancreatic EndoC-βH1 cells. EndoC-βH1 cells were infected at an MOI of 1 of the indicated recombinant CVB3 and cells were analyzed 24 h later. (A) Determination of virus titers by plaque assay. Fold changes between individual samples are shown. Mean values \pm SEM are displayed from two experiments performed each in duplicate. Significance: *** $P < 0.001$. (B) Determination of CVB3 VP1 and cellular proteins eIF4G, cleaved eIF4G, PARP, cleaved PARP, and cleaved Caspase 3 by western blotting. γ -tubulin was used as internal loading control. (C) Cytotoxicity of miR-375TS-containing CVB3. EndoC-βH1 cells were infected at an MOI of 1 of the indicated viruses. Cytotoxicity was determined by XTT assay 48 h after infection. Values measured for untreated cells were arbitrarily set to 100 and used to calculate the relative cytotoxicity of virally infected cells. Significance: **** $P < 0.0001$. n.s., not significant. Mean values \pm SEM are displayed from three experiments out of a total of eight samples.

rCVB3.1 (21.7-fold in Caco-2 and 7.1-fold in DLD-1) (Fig. 5A,B). Regarding cytotoxicity, Caco-2 cells were moderately susceptible to lytic infection with CVB3-375TS(3+) but completely resistant to CVB3-375TS(5+). However, the strength of cytotoxicity induced by CVB3-375TS(3+) was lower than for the parental rCVB3.1. In DLD1 cells, only weak cell lysis was observed for rCVB3.1, whereas the cells were completely resistant to CVB3-375TS(3+) and CVB3-375TS(5+) (Fig. 5A,B). The data indicate that the replication and the ability to

induce cytotoxicity in colorectal carcinoma cells are maintained in both CVB3 equipped with miR-375TS. However, compared to the parental rCVB3.1, CVB3-375TS(3+) shows slightly and CVB3-miR375TS(5+) moderately impaired performance. As lower replication and cytotoxicity were also seen in miR-39TS control viruses (Fig. 5A,B), the impairment of the miR-375TS viruses seems to be not induced by a specific interaction of miR-375TS with miR-375, which is expressed only at low levels in both cell lines (Fig. 1A).

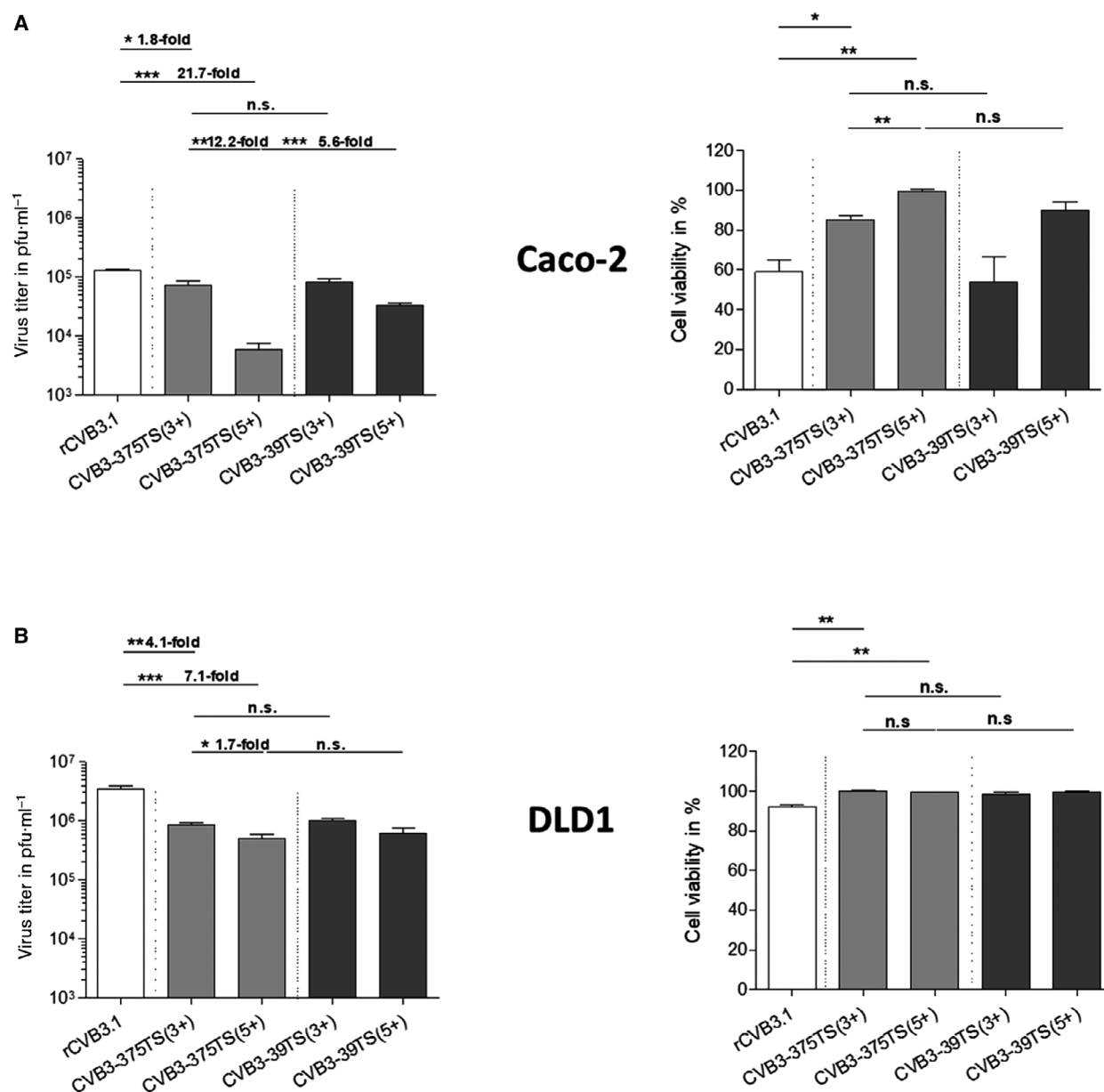


Fig. 5. Replication and cytotoxicity miR-375TS-containing CVB3 in the colorectal carcinoma cell lines. (A) Replication and cytotoxicity in Caco-2 cells. Caco-2 cells were infected at an MOI of 0.01 with the indicated viruses. Left—virus titers were measured in cell supernatants by plaque assay 48 h after infection. Right—cytotoxicity was determined by XTT assay 72 h after infection as described under Fig. 4C. Values measured for untreated cells were set as 100 and used to calculate cytotoxicity of virus-infected cells. Mean values \pm SEM are displayed from two experiments each performed in duplicate. (B) Replication and cytotoxicity in DLD1 cells. DLD1 cells were infected at an MOI of 100 with the indicated viruses. Left—virus titers were measured in cell culture supernatants 48 h after infection. Right—cytotoxicity was determined by XTT assay 144 h after infection, as described under A. Fold changes between individual samples are shown. Significance: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. n.s., not significant. Mean values \pm SEM are displayed from two experiments each performed in duplicate.

Discussion

CVB3 has potential as a new oncolytic virus for the treatment of cancer. However, with exception of the CVB3 variant PD [10,15], all CVB3 strains analyzed

so far also replicate in normal tissues, particularly in the pancreas and the heart, where they induce tissue injury and inflammation [13–15]. MiR-mediated virus detargeting represents a powerful technique to prevent

oncolytic viruses from replicating in nontargeted tissues, which makes this technique a valuable approach to increase the safety of this type of cancer therapy [23,34,35]. Addressing prevention of undesirable CVB3 replication in the heart by insertion of muscle-specific miR-206TS and miR-133TS has been carried out successfully [28], whereas attenuation of CVB3 in the pancreas and heart was achieved by insertion of miR-34aTS into the CVB3 genome [14].

Our research focus is on the development of oncolytic CVB3 for treatment of colorectal carcinomas [15]. Unfortunately, and in agreement with previous studies [36,37], we found that miR-34a was highly expressed in colorectal carcinoma cell lines, suggesting that a CVB3 equipped with miR-34aTS would probably be detargeted not only in the pancreas and the heart, but also in the colorectal cancer cells, which would negatively impact the outcome of the cancer treatment. As miR for cardiac-specific attenuation of CVB3 has already been defined [28] and may be easily used in the context of oncolytic CVB3, here we focused on the development of pancreas-attenuated CVB3. Therefore, CVB3 was equipped with miR-TS complementary to the miR-375, which we found is highly expressed in the pancreas but is weakly expressed in colorectal carcinoma cell lines (Fig. 1A). We show that CVB3 variants engineered with miR-375TS were highly susceptible to the pancreas-specifically expressed miR-375. Virus replication was drastically reduced by several orders of magnitude (up to 38 000-fold) in pancreatic cells expressing the miR-375 endogenously, whereas virus replication and cytotoxicity were largely retained in colorectal cancer cell lines. Moreover, by evaluating sites for integration of miR-375TS, our data reveal that both the 3'UTR and, as shown here for the first time, the protein coding region of the viral genome are suitable regions to make the virus sensitive to the miR-375.

Despite the fact that our data indicate that insertion of miR-TS into the viral genome *per se* slightly impairs virus replication and cytotoxicity, the site of miR-TS insertion is an additional crucial factor affecting silencing of the virus by the corresponding miRs. The genomic structure of picornaviruses can be divided into a 5'-UTR, the protein coding region, and a 3'UTR, and previously it was shown that CVB3 with miR-TS in the 5'-UTR and in the 3'UTR are susceptible to their corresponding miRs [14,23,27]. On the other hand, it has also been shown that certain sections within the 5'-UTR and 3'UTR do not tolerate miR-TS insertion, most likely because insertion of miR-TS disturbed higher-order RNA structures of the viral genome [28]. In the present study, we pursued a new approach and

inserted the miR-375TS into the 5' terminus of the polyprotein coding region of the CVB3 genome and compared it to insertion immediately downstream of the polyprotein stop codon in the 3'UTR, which is known to tolerate miR-TS [14]. Using the respective CVB3 cDNA constructs, the recombinant viruses were generated successfully, demonstrating that insertion of the miR-375TS at the selected sites was tolerated by the viruses. However, when comparing the suppression in miR-375 expressing pancreatic cells, CVB3 containing miR-375TS in the 3'UTR was suppressed far more than in CVB3 containing miR-375TS at the 5' terminus of the polyprotein coding region. Although the underlying mechanism remains to be elucidated, it is likely that the different sequences and resulting secondary structure flanking the miR-375TS sequence may influence the binding, stability, or the activity of the RNA-induced silencing complex (RISC), which is important for degradation of the viral genome.

Because of the resistance of the investigated colorectal carcinoma cell lines against the strain rCVB3.1, a reliable assessment regarding the differences in cytotoxic activity of miR-375TS viruses in the cancer cells was not possible. However, our data reveal that both viruses replicated in colorectal carcinoma cells, but the performance of the CVB3 with miR-375TS in the 3'UTR was better. On the other hand, this viral construct was not as effective as its parental rCVB3.1. In contrast to our data, other studies found very similar replication of parental and miR-TS-bearing oncolytic picornaviruses [14,27,28,35]. We can exclude suppression of miR-375TS by the rarely expressed miR-375 in colorectal carcinoma cells, since the miR-39TS control viruses did not replicate as well, suggesting that individual differences to miR-TS and the surrounding sequences used in our study may play a role in the lower activity we observed.

After infection, CVB3 generates a minus-strand RNA intermediate, from which multiple copies of viral plus-strand RNA copies are transcribed [38]. It has been shown that in infected cells there are far more plus strands than minus strands [39], which makes targeting of the minus-strand antigenome attractive for a miR-detargeting strategy. Our data demonstrate that the minus strand of CVB3, when equipped with miR-375TS, cannot be targeted by the corresponding miR-375. This observation is in line with an earlier report by Schubert *et al.* showing failure of inhibition of CVB3 replication when the viral minus strand was targeted by an siRNA [40]. It should be noted that a previous study found some reduction of viral replication in a miR-142TS containing CVA21 when targeting the minus-strand antigenome. However, compared to

targeting the plus-strand sense genome, the efficacy was 1000-fold lower in this study [35].

Evolution of viruses leads to an optimized viral life cycle. MiR-TS, when inserted into the virus genome, counteracts this process and therefore evolutionary pressure will work toward inactivating the miR-TS. There are two principal mechanisms through which the inhibition of the additional sequences can be countered by picornaviruses, either by acquiring mutations within the miR-TS [23] or by elimination of the miR-TS through homologous genomic recombination [41]. In tumor-bearing mice which had been treated with miR-regulated CVA21 for an extended period of time, mutations were detected but not deletion of the miR-TS [23,35], indicating that mutations are more easily acquired than deletion of the miR-TS. Interestingly, the mutations did not or only slightly affected the sensitivity of the virus for the miR [23], probably as multiple copies of a miR-TS were inserted into the viral genome and only single copies were mutated. As a consequence of these experiences, we inserted three copies of miR-375TS into our viruses in order to reduce the risk of development of viral escape mutants.

Unfortunately, the miR-375TS CVB3 described here showed only weak replication and cytotoxicity in colorectal carcinoma cells, which makes it unsuitable for further development as an oncolytic virus for the therapy of colorectal carcinomas. Recently, we investigated other CVB3 strains (Nancy, 31-1-93, H3, and PD) for their oncolytic potential in colorectal carcinomas [15]. All of them had distinct antitumor effects, but also induced side effects, including severe damage to the pancreas. Equipping these viruses with miR-375TS may improve their safety and thereby make them candidates for the therapy of colorectal cancer. Studies are underway to prove it.

In conclusion, we demonstrate here that insertion of miR-375TS into the genome of CVB3 attenuates the virus in pancreatic cells while preserving its ability to replicate in colorectal carcinoma cells. Moreover, our study reveals that the 3'UTR and the 5' terminus of the polyprotein protein coding sequence of CVB3 are suitable sites for the insertion of miR-TS, with insertion into the 3'UTR enabling stronger attenuation of the virus in the nontargeted pancreatic cells.

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Author contributions

MP constructed the viruses, performed virus growth experiments in HeLa cells and miR-transfection experiments. AH performed miR-transfection experiments and measured expression of miRs. KK and MS performed experiments in the pancreas cell line. BD performed experiments with colorectal carcinoma cell lines and virus growth experiments in HeLa cells. BT and JK assisted in writing the manuscript. SP and HF planned the experiments and wrote the manuscript.

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